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Long-term cryopreservation of embryogenic Scots pine cultures

Latutrie, Mathieu [1] and Aronen, Tuija [1]

Introduction

Somatic embryogenesis (SE) is considered the most potential vegetative propagation method for Scots pine (*Pinus sylvestris* L.). The existing SE protocols are based on the use of immature zygotic embryos as explants. Using explants like those, the final performance of the SE clones can be evaluated only by field tests of regenerated plants taking 5-10 years at the minimum. The Scots pine SE cultures have been found to gradually lose their embryo production capacity under continuous *in vitro* culture. Cryopreservation of Scots pine SE cultures is thus a prerequisite for successful clone delivery, being able to maintain regeneration ability during field testing.

Cryopreservation techniques have been developed for Scots pine SE cultures, but there are no reports on long-term cryostorage. The aim of the present work was to study potential effects of the long-term cryopreservation and different cryoprotectants on the viability, proliferation ability or embryo maturation capacity of Scots pine SE lines.

Materials and methods

Altogether 112 different SE lines from 4 donor trees were used material. The samples were cryopreserved according to Häggman et al. (1998) using either PDG mixture 1:1 or 1:2½ (108 lines), or DMSO solely as cryoprotectant (4 lines), and stored in liquid nitrogen for 2 to 14 years (from 1995-2007 to 2009). Following thawing and microscopical examination (Fig. 1) of the cryopreserved samples, proliferation and maturation experiments were performed according to slightly modified methods by Lelu-Walter et al. (2008), and the embryos germinated according to Aronen et al. (2009), (Fig. 2).

Results

At least 80% of the SE lines cryopreserved with PDG mixtures in 1999, 2001, and 2007 remained viable and started proliferating following thawing. The recovery was significantly lower for the lines cryopreserved with PGD in 1997 (Table 1). The lines preserved in 1995 using DMSO did not recover, and were thus excluded from further experiments.

The PDG mixture used and cryostorage time did not impact significantly the growth ratio of the SE lines, W1/W0 in 6-week proliferation varying 19-24x. At the maturation phase, the length of cryopreservation affected significantly the production of cotyledonary embryos, the quantity of embryos being lower in the lines cryopreserved for 8-12 years than in the ones cryostored for two years (Tables 2, 3). The PDG mixture and the donor trees had no significant effect on embryo production capacity.

Table 3: Production of cotyledonary somatic embryos per gFW in the SE lines cryopreserved in 1997-2007 and thawed in 2009. Significant differences among cryostorage times according to Student-Newman-Keuls post hoc test, $p<0.05$.

Cryostorage year	*Observations*	*Min - Max*	*Mean*	*Standard error*	*Groups*
1997	9	3 – 168	55,2	21,2	A
1999	27	0 – 490	59,4	21,6	A
2001	26	0 – 683	68,2	67,4	A
2007	19	0 – 977	292,0	71,5	B

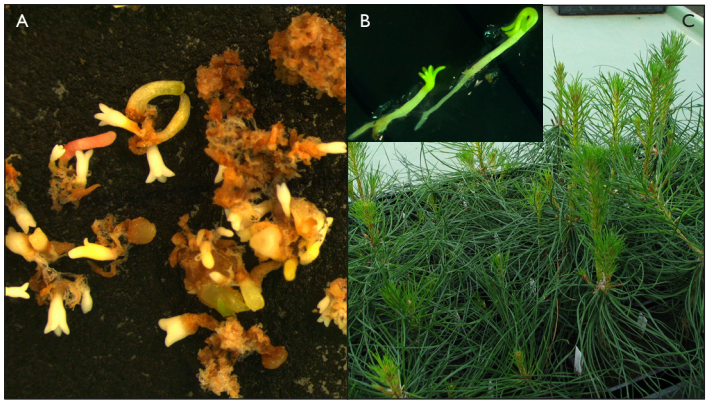

Fig. 2. Maturation (A), germination (B) and greenhouse establishment (C) of cryostored materials

Conclusions

Most of the Scots pine SE tissues remained viable after long-term cryopreservation with the PGD I or II used as cryoprotectant, but the regeneration was not possible with the DMSO treatment. The length of conservation, the PGD mixture used or the donor tree did not affect the proliferation of the SE-lines. The quantities of mature embryos produced, however, were dependent on the length of cryopreservation: the longer the time, the smaller the number of embryos. In addition to preservation time, also other factors may have affected the result, like the genotypic variation and original quality of the SE lines at the time of cryostorage, the SE technique for Scots pine being much enhanced (Lelu-Walter et al. 2008; Aronen et al. 2009) since the establishment of the oldest cryopreserved cultures.

Table 1: Viability and nature of the cryopreserved samples immediately after thawing together with the percentage of proliferating tissues after 9- 12 weeks of culture: + = positive (viable or embryogenic); - = negative (dead or not embryogenic); ? = undetermined

Cryopreservation year / *Cryoprotectant used*	*1997 PGDI*			*1999 PGDI*			*2001 PGDII*			*2007 PGDI*		
Number of SE - lines	*n=23*			*n=33*			*n=29*			*n=23*		
Observation	+	?	-	+	?	-	+	?	-	+	?	-
Viability (%)	91,3	4,3	4,3	90,9	9,1	0	93,1	6,9	0	91,3	8,7	0
Nature of the tissues (%)	78,3	21,7	0	90,9	9,1	0	96,6	3,4	0	100	0	0
Proliferation after 9 -12 weeks (%)	43,5	0	56,5	93,3	0	6,7	79,7	20,3	0	82,6	0	17,4

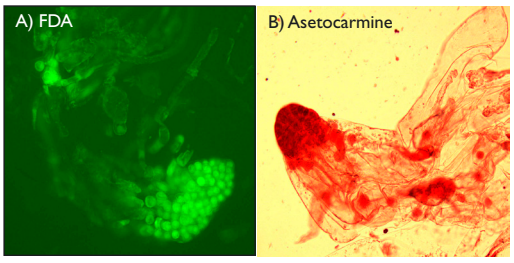

Fig. 1. FDA (A) and asetocarmine (B) staining was used for evaluation of viability and embryogenic nature of cultures

Table 2: Results of variance analysis for proliferation and maturation results

		Proliferation		*Maturation*	
Source of variation	*Degrees of freedom*	*F*	*p*	*F*	*p*
Year	2	2,04	0,12	7,34	0
Cryoprotectant used	1	3,13	0,08	0,06	0,81
Donor tree	2	0,46	0,64	0,82	0,45

References

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[1] Finnish Forest Research Institute, Punkaharju Research Unit, Finlandiantie 18, FI-58450 PUNKAHARJU, FINLAND
mathieu.latutrie@etudiants.purpan.fr, tuija.aronen@metla.fi

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